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Epithelial Cells in Nipple Aspirate Fluid

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13. ABSTRACT (Maximum 200 Words) We have successfully developed a method to isolate free DNA from ductal lavage (DL) and nipple aspirate fluid (NAF), and to use the isolated DNA for mitochondrial DNA (mtDNA) mutation analysis and LOH studies in order to improve early detection of breast cancer. We evaluated 26 DL and six NAF samples from 14 women of known <i>BRCA1</i> status (9 carriers and 5 non-carriers), with no clinical evidence of breast tumors. LOH studies at the <i>BRCA1</i> locus were possible in 19/26 DL samples, and at the <i>FHIT</i> locus in 16/26 samples. In 4/9 mutation carriers we found LOH at the <i>BRCA1</i> allele, and in 2 of these we also found LOH at the <i>FHIT</i> allele. In one of the mutation carriers with <i>BRCA1</i> LOH, invasive breast cancer was subsequently detected, and the tumor showed the same LOH as the DL. In one of the true negatives, <i>BRCA1</i> and <i>FHIT</i> LOH were detected. The mitochondrial studies were possible in all 26 DL samples and a somatic mutation at the D310 marker was found in 3/9 carriers, 2 of whom also had LOH at the <i>BRCA1</i> locus, and in none of the non-carriers. mtDNA mutation evaluation was possible in 4/6 NAF samples. The NAF and DL results were concordant. One NAF sample from a <i>BRCA1</i> patient showed a mtDNA mutation. Our data demonstrates the feasibility of molecular studies using the free DNA present in the ductal fluid, while the intact cells can be used for cytologic studies.				
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Introduction:

The optimal method for early detection of breast cancer in premenopausal women is not known. Currently, mammographic screening is the best available approach for early detection in the general population. However, mammography alone may not be sufficient in young women, for the following reasons: (a) effectiveness of mammography has not been established in women younger than 40 since younger women have more dense breast tissue, which reduces mammographic sensitivity [1], (b) tumor growth rates may be higher in younger women thereby necessitating frequent screening, and (c) carriers of some mutations (such as ataxia telangiectasia) may have increased sensitivity to radiation and conceivably could be harmed by frequent mammograms [2]. Therefore, there is a critical need to develop screening methods to augment mammography for the early detection of breast tumors, particularly in high-risk women, such as *BRCA1* or *BRCA2* mutation carriers. Women inheriting a mutation in either *BRCA1* or *BRCA2* face elevated lifetime risks of breast cancer with estimates of risk varying between 37-85% by age 70 [3, 4]. Typically, these cancers occur at a younger age than is seen in the general population, with studies demonstrating up to 50% of mutation carriers being diagnosed with breast cancer by age 50 [4]. In addition, mutation carriers who have already been diagnosed with breast cancer face a significantly elevated risk of developing a second primary. Whereas women with sporadic breast cancer have been estimated to have a 0.5-1.0% annual risk of developing contralateral breast cancer (5-10%/10 year risk), mutation carriers have been found to have about a 35% 10 year risk of contralateral breast cancer [4, 5].

Given the high risk of breast cancer in mutation carriers, the Cancer Genetics Studies Consortium, a consortium convened by the National Human Genome Research Institute, has recommended that *BRCA1/2* carriers, and those with family histories consistent with hereditary breast cancer, begin to perform breast self exam between the ages of 18-21, have clinician-performed examinations every 6-12 months beginning between the ages of 25-35, and have mammograms performed on an annual basis, beginning between the ages of 25 and 35 [6]. Similar guidelines have been published by other groups [7, 8]. The French National Ad Hoc Committee has also recommended that MRI should be evaluated in the context of clinical trials, but that MRI should never be used alone for screening purposes.

The high cancer risk in mutation carriers, combined with the lack of known benefit from the currently recommended cancer screening options, make this an ideal population in which to study new breast screening technologies. *One approach would be the use of molecular markers associated with neoplastic changes in a screening strategy for early detection.*

It is widely believed that the accumulation of genomic aberrations occurs very early in the process of mammary tumorigenesis and may precede morphologic changes [9, 10, 11]. Therefore, development of methods which permit the reliable identification of these early genetic changes in mammary cells obtained through non-invasive or minimally invasive approaches such as ductal lavage or nipple aspiration, will greatly benefit early detection of breast cancer.

Studies have shown that LOH at relevant polymorphic markers is present in hyperplasias (usual ductal hyperplasia and atypical ductal hyperplasia) from both

cancerous and noncancerous breasts [12, 13, 14], as well as in morphologically normal lobules, adjacent to sporadic breast cancer [10, 11]. In a recent study supported by an Idea Award from the DOD (DAMD17-99-1-9193), we have demonstrated the presence of LOH in morphologically normal tissues and in benign tissues from breast cancer patients that are carriers of mutations in the *BRCA1* and *BRCA2* genes [11]. Such changes may represent the earliest detectable genomic aberrations that occur during the development and progression of breast cancer in these high-risk patients. Our conclusion from that study was that LOH at the relevant *BRCA* loci is an early event in *BRCA* mutation carriers, and may be detected in non-malignant cells [11]. Recent studies have also shown the presence of several mitochondrial DNA (mtDNA) mutations in a variety of tumor types [15]. Furthermore, these mitochondrial mutations were shown to be readily detectable in the bodily fluids [16]. A mtDNA mutation at the microsatellite marker D310 was reported in about 30% of breast cancers [17], and was detectable in breast fine needle aspirate fluid [18]. *Taken together, a strategy allowing the detection of LOH at relevant markers or mtDNA mutations in the ductal fluid samples will provide an important early detection approach that could complement conventional screening. This constitutes the basis of our current project.*

Nipple aspirate fluid (NAF) has been studied for many years as a non-invasive method to examine changes in breast biology and to identify women with high risk of breast cancer or preclinical disease [19]. NAF is secreted continuously by the non-lactating breast and, in 50-70% of premenopausal women, it can be aspirated through ductal openings in the nipple using a simple, non-invasive pump. NAF is of interest because it has a relatively long retention time in the breast alveolar-ductal system where it accumulates exfoliated mammary epithelial cells [19, 20, 21]. Evaluating these cells may provide not only a "snapshot" of the micro-environment where breast cancer originates, but also a realistic opportunity to improve the detection of breast tumors at their earliest stages. However, a major hurdle impeding the success of NAF based assays is the low cellularity and the low volume of NAF samples. The typical NAF sample from a woman with no breast abnormalities is about 10 μ l in volume and contains fewer than 10 ductal epithelial cells [19]. One very promising new approach to overcoming these two issues has recently been designed and tested: Breast Ductal Lavage.

Ductal lavage [22] is a new, minimally invasive technique developed to evaluate the ductal fluid and cells. A small catheter is inserted into the duct. Saline is instilled and the breast is massaged to dislodge cellular material lining the duct. The fluid is then collected for analysis. In a recent study [22], ductal lavage was performed on greater than 500 high-risk women at 19 breast cancer centers. Atypical cells were seen in 17% of patients and suspicious or malignant cells were identified in 7% of patients. The lavage procedure also resulted in specimens sufficient for cellular analysis in a far greater number of patients than did nipple aspiration; in fact, ductal lavage produced an average of 40,000 cells per duct. This high yield of cells makes it much more likely that sufficient cells would exist for both cytologic analysis and for genetic studies. In addition, this procedure allows the physician to pinpoint the specific duct from which the abnormal cells originated, so that a specific area of the breast can be more thoroughly evaluated and more closely followed.

This project allowed us to develop an approach to isolate free DNA from the lavage fluid, thereby leaving the entire cellular content for cytological analysis. This

approach offers the distinct advantage of allowing the cytologist to evaluate all the recovered cells, while enough free DNA can be isolated and used for PCR based assays. We have also successfully isolated free DNA from the NAF fluid for molecular evaluation by PCR. The isolated DNA was used in genomic and LOH studies and to detect mitochondrial DNA (mtDNA) mutations. Both LOH at relevant polymorphic markers [14, 23, 24], and mutations of mtDNA [16, 17, 18] are frequently detected in tumors and could serve as markers of high risk, premalignancy, or of tumorigenesis itself in mammary epithelial cells. We have recently published our findings in *Breast Cancer Research and Treatment* [25].

Body:

In this project, we investigated the possibility of using the ductal lavage fluid and the nipple aspirate fluid to detect abnormalities in molecular markers which can be used as an early indicator of breast tumorigenesis.

Study participants were accrued through the Familial Cancer Registry (FCR), a shared resource of the Lombardi Comprehensive Cancer Center (LCCC). The FCR includes over 1300 individuals with familial or hereditary breast cancer. Members of the FCR complete questionnaires, which include detailed information on their medical history, family history, breast and ovarian cancer risk factors, and other comprehensive epidemiological data. These data are updated on an annual basis. In addition, if these women have undergone any surgery, be it prophylactic or for diagnostic or treatment purposes, the tissue is obtained and stored. Blood is also obtained from these patients and the lymphocytes are immortalized.

In this project, the following methods were developed and evaluated:

A method to isolate and use the free DNA present in NAF and DL:

We developed an approach that allows the isolation of free DNA from the lavage fluid, thereby leaving the entire cellular content for cytological analysis. This approach offers the distinct advantage of allowing the cytopathologist to evaluate all the recovered cells, while enough free DNA can be isolated and used for PCR based assays. We have also successfully isolated free DNA from the NAF fluid for molecular evaluation by PCR. The isolated DNA was used to detect mitochondrial DNA (mtDNA) mutations. It was also used in LOH studies at relevant polymorphic markers.

mtDNA mutation analysis in DL and NAF samples:

We have optimized a method to detect mitochondrial DNA mutations in free DNA isolated from DL and NAF, as an early sign of breast cancer. We have initiated an active collaboration with Dr. David Sidransky and his group at Johns Hopkins University to study mtDNA mutations in the ductal fluid. Dr. Sidransky's group discovered that mtDNA mutations at the microsatellite marker D310 in the DL and NAF fluids is present in over 30% of breast cancers [17]. Our working hypothesis is that women with early-

stage breast cancer may have mtDNA mutations in mammary epithelial cells shed into the nipple aspirate fluid (NAF) and the ductal lavage (DL) fluid which can be used as a marker for screening and detection of early disease. In addition, we hypothesize that mtDNA mutations in NAF and DL-derived cells are representative of those found in tumor tissue in the same breast. Through our collaboration with Dr. Sidransky, we were successful to set up the assay for the detection of mutations at the D310 marker in the DL and the NAF fluids.

We have performed ductal lavage on a total of 14 subjects: nine *BRCA1* carriers and five true-negatives. Of these, seven had adequate material for cytologic analysis and only one, a *BRCA1* mutation carrier, demonstrated focal minimal atypia. The remainder had benign findings. A total of 26 DL specimens were obtained [Table 1].

The DL specimens were evaluated for mitochondrial DNA mutations at the microsatellite marker D310, located at the D-Loop region using a PCR based assay. The mitochondrial studies were possible in all 26 DL samples. In three of the carriers, D310 length difference between the DL samples and the corresponding blood sample (somatic mutation) was found. No mtDNA mutations were detected in the true-negative subjects. Figure 1a shows examples of DL samples with no mtDNA mutations and Figure 1b shows an example of a DL sample with a mtDNA mutation. We also evaluated six NAF samples from four patients (two carriers and two non-carriers) for mitochondrial mutations. The evaluation was possible in four of the six samples. A mutation was detected in one sample from a carrier whose DL from the same duct also showed the same mutation (Figure 1b). Three samples had no evidence of mutations, and no mutations was seen in their corresponding DL samples either. In all four NAF samples, the mitochondrial DNA findings were consistent with the corresponding DL findings. The patients with mitochondrial abnormalities in the DL fluid had normal physical exam, a normal mammogram and normal findings on FDG-PET scan. The patients continue to be closely monitored.

LOH analysis of the DL and NAF fluids:

Because of the very exciting results that we obtained from our other DOD funded project [11], namely the detection of genetic changes in the form of LOH in morphologically normal mammary tissues and in benign tissues from breast cancer patients that are carriers of mutations in the *BRCA1* and *BRCA2* genes, we performed LOH analysis at the *BRCA1* and *FHIT* loci, on the same DL and NAF fluid specimens used for the mitochondrial studies. *The ability to conduct more than one molecular assay on the same fluid sample offers a tremendous advantage for early detection of breast cancer, as it increases the chances to detect an early molecular change which in turn may indicate the presence of an early cellular transformation.*

LOH studies at the *BRCA1* locus were possible in 19 of 26 DL samples, and at the *FHIT* locus in 16 of 26 samples. In 4 of the 10 mutation carriers we found LOH at the *BRCA1* locus, and in 2 of these we also found LOH at the *FHIT* locus. In one of the mutation carriers with LOH at the *BRCA1* and *FHIT* loci, minimal cytologic atypia was noted. This patient was diagnosed with invasive breast cancer in the contralateral breast, and is

described in details later in this section. In the other 3 carriers, either few cells were obtained, and thus the specimen was inadequate for cytologic evaluation, or the ductal lavage yielded cytologically benign cells. In one of the true negatives LOH at *BRCA1* and *FHIT* loci were also seen. Figure 2 shows an example of a DL case with LOH at the *BRCA1* and the *FHIT* genes.

In one *BRCA1* carrier with a recent unremarkable, mammogram and physical exam, FDG-PET scan demonstrated a focal area of increased uptake in her right breast. This was subsequently biopsied and found to be an invasive and non-invasive ductal carcinoma. In this carrier, LOH at the *BRCA1* locus was seen in DL specimens obtained from both her right (affected) and left breast. The DL specimen from her right breast had insufficient material for cellular analysis. On the left, focal minimal atypia was seen. The patient underwent bilateral mastectomy. LOH studies of the tumor tissues showed LOH at the *BRCA1* locus as was seen in the DL sample from the same breast. Figure 3 shows LOH studies at the *BRCA1* locus performed on the DL fluid and on the breast tumor. Concordance between the 2 studies was noted. No evidence of mitochondrial DNA mutations was detected in the tumor or the DL samples from this patient. Careful evaluation of the non-affected breast by the pathologist showed no abnormalities. The other patients with LOH or mitochondrial abnormalities in the DL fluid had normal physical exam, a normal mammogram and normal findings on FDG-PET scan. The patients continue to be closely monitored. Of note, we were able to perform the LOH analyses and to evaluate mtDNA mutation in participants for whom ductal lavage yielded adequate cellularity, and more interestingly, in those in whom the ductal lavage was acellular. These findings indicate that the acellular specimen can still yield worthwhile and informative material for analysis. Table 1 summarizes the results of the whole study.

Table 1: Results of LOH and mtDNA studies in the DL fluid.

Patient	Age (Years)	DL Samples	BRCA1 Status	mtDNA Mutation	BRCA1 LOH	FHIT LOH	Cytology
1	58	1	POS	mut	NR	NR	Acellular
2	58	1 ^b	POS	mut	LOH	No LOH	Normal
3	33	1 ^c	POS	No mut	NR	NR	Acellular
		2 ^c		No mut	No LOH	No LOH	
		3		No mut	No LOH	No LOH	
		4		No mut	NR	NR	
4	34	1	POS	No mut	No LOH	No LOH	Acellular
		2		No mut	No LOH	No LOH	
5	36	1	POS	No mut	No LOH	No LOH	Acellular
6	31	1	POS	mut	LOH	LOH	Acellular
		2		mut	LOH	NR	
7	28	1	POS	No mut	LOH	NR	Normal
		2		No mut	LOH	NR	
8 ^d	45	1	POS	No mut	LOH	LOH	Focal Minimal Atypia
		2		No mut	LOH	NR	Acellular
9	59	1	POS	No mut	NR	NR	Acellular
10	53	1 ^a	NEG	No mut	No LOH	No LOH	Normal
11	57	1	NEG	No mut	LOH	LOH	Normal
12	47	1 ^a	NEG	No mut	No LOH	No LOH	Normal
		2 ^a		No mut	No LOH	No LOH	
		3		No mut	No LOH	No LOH	
13	35	1	NEG	No mut	No LOH	NR	Acellular
		2		No mut	No LOH	NR	
14	35	1	NEG	No mut	NR	No LOH	Normal
		2		No mut	NR	No LOH	
		3		No mut	NR	No LOH	

^a indicates that the corresponding NAF was studied and did not show a mutation

^b indicates that the corresponding NAF was studied and showed a mutation

^c indicates that the corresponding NAF was studied and no results were obtained

^d patient with cancer

NR= No result

POS= mutation carrier, NEG= non-carrier

mut= mutation

Figure 1:

Analysis of the D310 marker in DL samples from 2 different patients. Figure 1A shows the analysis of the D310 marker in 3 DL samples obtained from three different ducts from the same patient, a non-carrier of the *BRCA1* mutation (Lanes DL1, DL2, DL3). No mtDNA mutation was found. Lane N shows the D310 pattern in the blood. Figure 1B, shows a mutation (arrow) at the D310 marker observed in a DL sample (Lane DL) and in the NAF sample (Lane NAF) from another case, a *BRCA1* carrier. Lane N shows the D310 pattern in the blood of that patient.

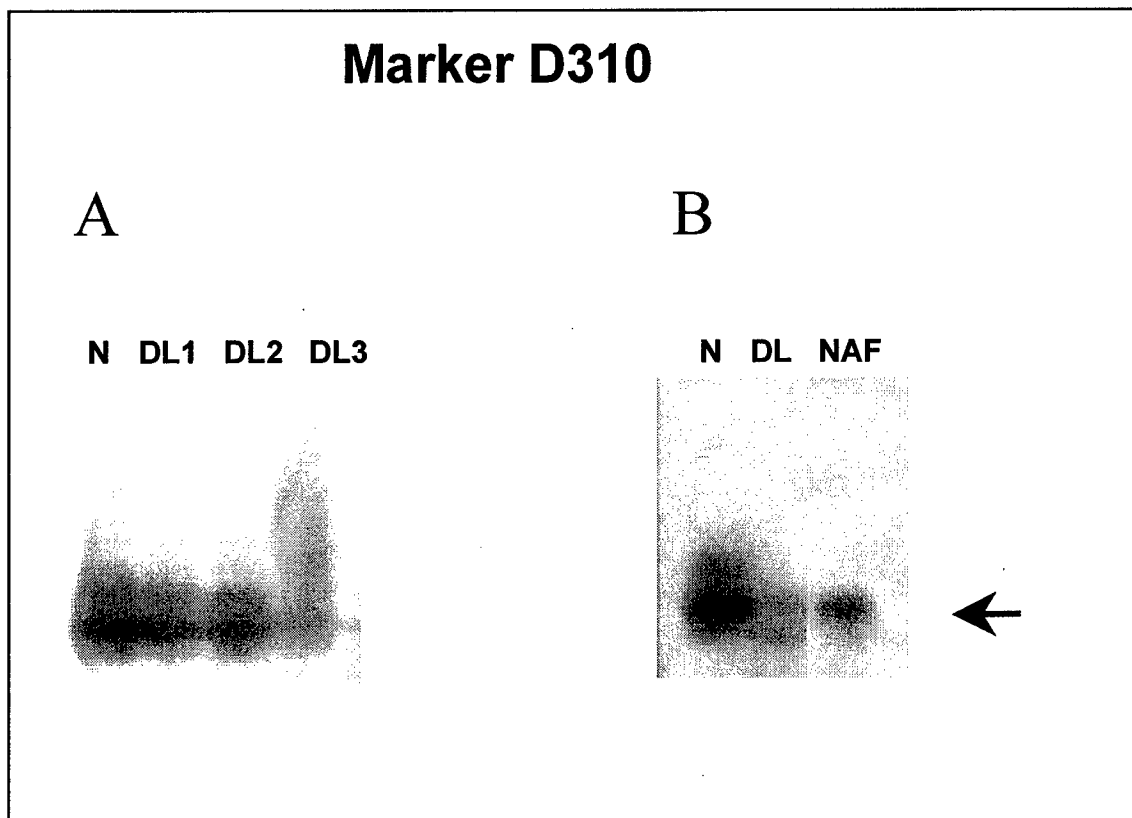


Figure 2:

LOH analysis at the *BRCA1* (top) and the *FHIT* (bottom) loci, in DL fluid from a *BRCA1* carrier, showing LOH at both loci.

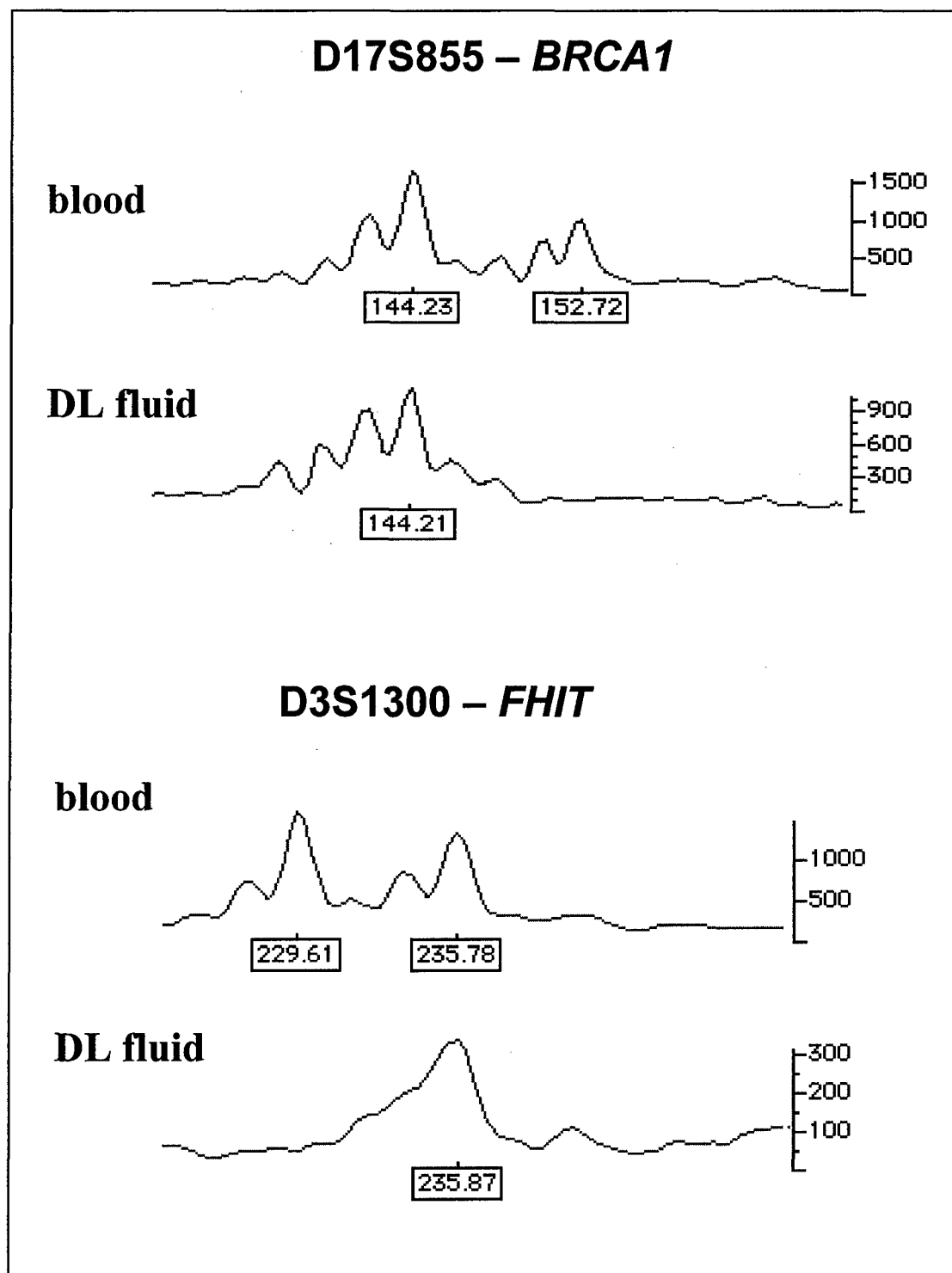
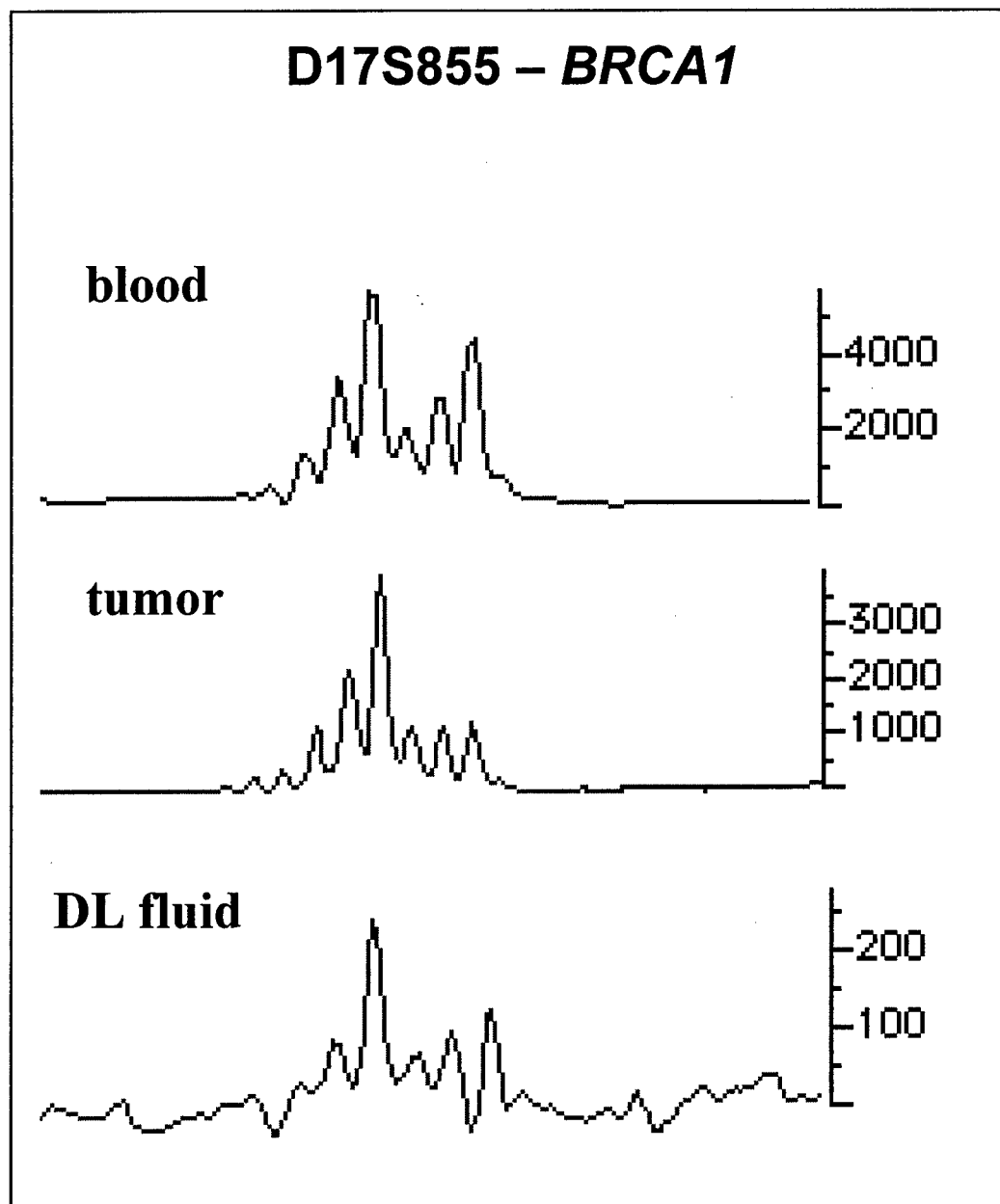


Figure 3:

LOH analysis at the *BRCA1* locus in the tumor and the ductal lavage fluid from the same breast showing the same LOH in both.



Key Research Achievements:

- Optimized conditions to isolate free DNA from ductal lavage and nipple aspirate fluid specimens.
- Optimized conditions to detect mtDNA mutations at the D310 marker in DNA isolated from DL and NAF.
- Optimized conditions to study LOH at 2 markers (*BRCA1* and *FHIT*) in DNA isolated from DL and NAF.
- Evaluate 26 specimens from patients with known *BRCA1/2* status.

Reportable outcomes:

-Published manuscript:

Isaacs C, Cavalli LR, Cohen Y, Pennanen M, Shankar LK, Freedman M, Singh B, Liu M, Gallagher A, Rone JD, Dickson RB, Sidransky D, and **Haddad BR**. Detection of LOH and mitochondrial DNA alterations in ductal lavage and nipple aspirate fluids from high-risk patients. *Breast Cancer Research and Treatment* 84(2):99-105 (2004).

-Abstract and Poster presentation at the American Society of Human Genetics (ASHG) Annual Meeting, 2003:

Haddad BR, Cavalli LR, Shankar LK, Freedman M, Singh B, Pennanen M, Liu M, Gallagher A, Rone JD, Dickson RB, and Isaacs C. Strategies for Early Detection of Breast Cancer in High Risk Women Using Cytologic and Molecular Studies of Ductal Lavage Fluid.

Conclusions:

This study supported by a concept award (DAMD17-01-0525) demonstrates that free DNA can be isolated from the ductal lavage and nipple aspirate fluid and can be successfully used to evaluate molecular markers. Using the DNA we have investigated the presence of mtDNA mutations at marker D310 and also the presence of LOH at the *BRCA1* and *FHIT* genes in high-risk patients for breast cancer. *The ability to conduct more than one molecular assay on the same fluid sample offers a tremendous advantage for early detection of breast cancer, as it increases the chances to detect an early molecular change which in turn may indicate the presence of an early cellular transformation.*

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Personnel:

This project was completed in Dr. Haddad's lab at Georgetown University/Lombardi Comprehensive Cancer Center Researchers. Drs. Bassem Haddad (PI) and Luciane Cavalli (Post-Doctoral Fellow) mainly worked on the laboratory aspects of this projects. The projects involved collaborations with other investigators (not receiving pay from this grant).

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- Isaacs C, Cavalli LR, Cohen Y, Pennanen M, Shankar LK, Freedman M, Singh B, Liu M, Gallagher A, Rone JD, Dickson RB, Sidransky D, and Haddad BR. Detection of LOH and mitochondrial DNA alterations in ductal lavage and nipple aspirate fluids from high-risk patients. *Breast Cancer Research and Treatment* 84(2):99-105 (2004).

- Haddad BR, Cavalli LR, Shankar LK, Freedman M, Singh B, Pennanen M, Liu M, Gallagher A, Rone JD, Dickson RB, and Isaacs C. Strategies for Early Detection of Breast Cancer in High Risk Women Using Cytologic and Molecular Studies of Ductal Lavage Fluid. Presented at the American Society of Human Genetics (ASHG) Annual Meeting, 2003.

Appendix:

- Abstract presented at the American Society of Human Genetics (ASHG) Annual Meeting, 2003.

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Strategies for Early Detection of Breast Cancer in High Risk Women Using Cytologic and Molecular Studies of Ductal Lavage Fluid. Bassem R. Haddad, Luciane R. Cavalli, Lalitta K. Shankar, Matthew Freedman, Baljit Singh, Marie Pennanen, Minetta Liu, Ann Gallagher, Janice D. Rone, Robert B. Dickson, and Claudine Isaacs. Lombardi Cancer Center, Georgetown University, Washington, DC.

We have established a pilot study in which *BRCA1* carriers and true negatives with no clinical evidence of breast abnormalities, either by physical exam or mammogram, undergo ductal lavage (DL). The DL fluid is evaluated by routine cytology and then examined for molecular genetic abnormalities such as LOH. Previous work done by our group has demonstrated LOH of the *BRCA1* & *FHIT* wild type alleles in non-malignant breast tissues surrounding breast tumors, suggesting that these are early genetic change which could be used as markers for early detection. To date we have enrolled 30 patients in our study: 20 *BRCA1* mutation carriers and 10 controls. DL was attempted on 28 subjects and was successful in 15. Of those, 8 had adequate material for cytologic analysis and only 1, a *BRCA1* mutation carrier, demonstrated focal minimal atypia. The remainder had benign cytologic findings. DNA was extracted from the DL supernatant obtained from all 15 subjects for molecular studies. LOH analysis for the *BRCA1* and the *FHIT* genes was completed on 13 subjects, 9 carriers and 4 controls. In 4 of the 9 mutation carriers we found LOH at the *BRCA1* allele; and in 2 of these we also found LOH at the *FHIT* allele. In 1 of the true negatives LOH at *BRCA1* and *FHIT* loci were also seen. In 1 of the 2 *BRCA1* carriers with LOH at the *BRCA1* and *FHIT* loci, minimal cytologic atypia was noted. In the other 3 either few cells were obtained or the DL yielded cytologically benign cells. The patient with minimal atypia had a recent unremarkable mammogram and physical exam. Breast FDG-PET scan showed a focal area of increased uptake in her right breast. This was biopsied and found to have invasive ductal carcinoma. She underwent bilateral mastectomy. In this carrier, LOH at the *BRCA1* locus was seen in the DL fluid from both breasts. The DL specimen from her right breast (affected) had insufficient material for cellular analysis and focal minimal atypia was seen in the DL sample from the left breast. Accrual to this study is ongoing.



Report

Detection of LOH and mitochondrial DNA alterations in ductal lavage and nipple aspirate fluids from high-risk patients

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Summary

We describe a method for the isolation of free DNA from ductal lavage (DL) and nipple aspirate fluid (NAF), and its evaluation for the presence of LOH at the *BRCA1* and *FHIT* genes and for mitochondrial DNA (mtDNA) mutations at the D310 marker, to improve early detection of breast cancer. We evaluated 26 DL and six NAF samples from 14 women of known *BRCA1* status, who have no clinical evidence of breast tumors: nine mutation carriers and five non-carriers. LOH studies at the *BRCA1* locus were possible in 19/26 DL samples, and at the *FHIT* locus in 16/26 samples. In 4/9 mutation carriers we found LOH at the *BRCA1* allele, and in two of these we also found LOH at the *FHIT* allele. In one of the mutation carriers with *BRCA1* LOH, invasive breast cancer was subsequently detected, and the tumor showed the same LOH as the DL. In one of the true negatives, *BRCA1* and *FHIT* LOH were detected. The mitochondrial studies were possible in all 26 DL samples and a somatic mutation was found in 3/9 carriers, two of whom also had LOH at the *BRCA1* locus, and in none of the non-carriers. mtDNA mutation evaluation was possible in 4/6 NAF samples. The NAF and DL results were concordant. One NAF sample from a *BRCA1* patient showed a mtDNA mutation. Our data demonstrates the feasibility of performing molecular studies using the free DNA present in the ductal fluid, while the intact cells can be used for cytologic studies.

Introduction

In the general population, mammographic screening is currently the best available approach for early detection of breast cancer. However, the optimal screening regimen in the high-risk women is not yet known. Studies have demonstrated that mammography has a relatively lower sensitivity in younger women [1]. This lower sensitivity is presumed to be due to the greater breast radiodensity in this group. Early data on breast screening with other modalities, such as magnetic resonance imaging (MRI), ultrasound, and nuclear scanning, whose sensitivity appears largely independent of breast density, may be of further benefit

in these younger women [2]. Therefore, there is a critical need to develop screening methods to augment mammography for the early detection of breast tumors, particularly in high-risk women, such as *BRCA1* or *BRCA2* mutation carriers, who face markedly elevated risks of developing early onset breast cancer. One approach would be the use of molecular markers associated with neoplastic changes in a screening strategy for early detection.

Nipple aspirate fluid (NAF) has been studied for many decades as a non-invasive method to examine changes in breast biology and to identify women at high risk for breast cancer or preclinical disease, based on conventional cytologic detection of abnormal cells

in their NAF [3]. However, NAF cytology alone is not sufficiently sensitive to identify the subgroup of women who are on a progression pathway that will lead to breast cancer [4], particularly because very few epithelial cells are obtained using this procedure. More recently, a minimally invasive procedure, ductal lavage (DL), was developed in order to obtain breast ductal epithelial cells [5]. In this procedure, the individual duct is catheterized with a small microcatheter (Pro-Duct Health, CA) and flushed with up to 20 cc of saline to collect mammary epithelial cells from the entire ductal tree. This procedure allows the collection of thousands of epithelial cells that can be used for cytologic and molecular studies [5–8].

Here we describe an approach that allows the isolation of free DNA from the lavage fluid, thereby leaving the entire cellular content for cytological analysis. This approach offers the distinct advantage of allowing the cytopathologist to evaluate all the recovered cells, while enough free DNA can be isolated and used for PCR based assays. We have also successfully isolated free DNA from the NAF fluid for molecular evaluation by PCR. The isolated DNA was used in genomic and LOH studies and to detect mitochondrial DNA (mtDNA) mutations. Both LOH at relevant polymorphic markers [9–12] and mutations of mtDNA [13–15] are frequently detected in tumors and could serve as markers of high risk, premalignancy, or of tumorigenesis itself in mammary epithelial cells. Therefore, the ability to detect these changes in the DL fluid and NAF may provide an important addition to the detection techniques currently available for breast cancer. In this report, we studied LOH at the *BRCA1* gene and the *FHIT* gene; we also investigated the presence of mtDNA mutations at the mitochondrial microsatellite marker D310. The D310 marker is a mononucleotide repeat of 300–315 nt which is located at the D-loop region of mtDNA, and it is involved in the mtDNA replication process [15]. Mutations at this region are common in primary human tumors, including breast cancer where they have been reported to occur in about 30% of cases, and are likely to have functional relevance in tumor development [15].

Materials and methods

Patient population

As part of an IRB-approved protocol at the Lombardi Comprehensive Cancer Center (LCCC) at Georgetown

University, we enrolled women on a study evaluating the role of DL and PET scan as novel screening tools. In order to be eligible, women had to have had a normal breast exam and mammogram within 12 months of study entry. We evaluated specimens from 14 women of known *BRCA1* status, from high-risk families enrolled in the LCCC Familial Cancer Registry (Table 1). Nine subjects were *BRCA1* mutation carriers, and five were true negatives. A true negative is defined as an individual who has tested negative for the mutation known to be present in a close relative. If possible DL samples were obtained from both breasts and more than one sample may have been obtained from the same breast. In total we studied 26 DL samples from 14 individuals, for both LOH and mtDNA mutations; 16 from the *BRCA1* carriers and 10 from the true negatives. We also studied six NAF samples for the presence of a mtDNA mutation. Three NAF samples were from *BRCA1* carriers and three from non-carriers. Thirteen women had no evidence of breast tumor clinically or by radiologic evaluation (mammography and FDG-PET scan). One woman demonstrated a focal area of increased uptake by FDG-PET scan, despite a negative mammogram, and was found to have invasive breast cancer on biopsy. In that patient, DL fluid was obtained from the breast with tumor and from the contralateral breast. Tumor tissue was also obtained following surgery and was evaluated for LOH and mtDNA mutations. The findings from tissue analysis were compared to the DL analysis.

DL specimen handling and cytologic analysis

DL was performed as described earlier [5]. For each patient, the fluid was collected from each individual duct and kept separate (rather than being pooled with the fluid obtained from other ducts) and diluted in polypropylene tubes pre-filled with a preservative solution (e.g., Cytolyt®). The specimen was centrifuged at 1700 rpm for 10 min. The supernatant was set aside for DNA extraction. The sediment was transferred to a container filled with Preservcyt® solution. A 'thin prep' slide was prepared using a standard method and the slide was stained using Papanicolaou technique. Each slide was examined by the cytopathologist. In order to be deemed adequate for analysis, a slide must contain at least 10 epithelial cells. The cells were categorized as: (1) insufficient cellular material for diagnosis (i.e., less than 10 cells present); (2) benign epithelial cells; (3) mildly atypical epithelial

Table 1. Results of LOH and mtDNA studies in the DL fluid

Patient	Age (years)	DL samples	BRCA1 status	mtDNA mutation	BRCA1 LOH	FHIT LOH	Cytology
1	58	1	POS	Mut	NR	NR	Acellular
2	58	1 ^b	POS	Mut	LOH	No LOH	Normal
3	33	1 ^c	POS	No mut	NR	NR	Acellular
		2 ^c		No mut	No LOH	No LOH	
		3		No mut	No LOH	No LOH	
		4		No mut	NR	NR	
4	34	1	POS	No mut	No LOH	No LOH	Acellular
		2		No mut	No LOH	No LOH	
5	36	1	POS	No mut	No LOH	No LOH	Acellular
6	31	1	POS	Mut	LOH	LOH	Acellular
		2		Mut	LOH	NR	
7	28	1	POS	No mut	LOH	NR	Normal
		2		No mut	LOH	NR	
8 ^d	45	1	POS	No mut	LOH	LOH	Focal minimal atypia
		2		No mut	LOH	NR	Acellular
9	59	1	POS	No mut	NR	NR	Acellular
10	53	1 ^a	NEG	No mut	No LOH	No LOH	Normal
11	57	1	NEG	No mut	LOH	LOH	Normal
12	47	1 ^a	NEG	No mut	No LOH	No LOH	Normal
		2 ^a		No mut	No LOH	No LOH	
		3		No mut	No LOH	No LOH	
13	35	1	NEG	No mut	No LOH	NR	Acellular
		2		No mut	No LOH	NR	
14	35	1	NEG	No mut	NR	No LOH	Normal
		2		No mut	NR	No LOH	
		3		No mut	NR	No LOH	

^a Indicates that the corresponding NAF was studied and did not show a mutation.

^b Indicates that the corresponding NAF was studied and showed a mutation.

^c Indicates that the corresponding NAF was studied and no results were obtained.

^d Patient with cancer. NR = No result; POS = mutation carrier; NEG = non-carrier; Mut = mutation.

cells; (4) markedly atypical epithelial cells; or (5) malignant cells.

DNA extraction

Using the DNAeasy Tissue kit (Qiagen), DNA was prepared from the supernatant DL fluid. For the NAF, the fluid sample was placed directly in 10 µl of water and heated at 65°C for 10 minutes prior to PCR analysis.

LOH analysis

In order to detect evidence of LOH at the *BRCA1* and the *FHIT* gene loci, we used two intragenic dinucleotide polymorphic markers D17S855 (*BRCA1*) and D3S1300 (*FHIT*). Prior to the analysis of the LOH in the DL fluid, each microsatellite marker was first eval-

uated for informativeness and allele size using DNA prepared from the patient's peripheral blood. The PCR and cycling conditions were adapted for each primer set [16]. For each reaction, the genomic DNA obtained from the peripheral blood of the patient was included as normal control for LOH analysis. PCR was performed using a PTC-200 thermo-cycler (MJ Research, Waltham, MA). The primer sets were obtained from Invitrogen (Huntsville, AL). The forward primers for each set were labeled using one of two fluorescent dyes, HEX or FAM. Allele sizes were determined by electrophoresis of PCR products in 6% denaturing polyacrylamide gels and compared to ROX 500 size standards (Applied Biosystems, Foster City, CA), using an automated sequencer (ABI 377), according to manufacturer's instructions (Applied Biosystems, Foster City, CA). The fluorescent signals from the dif-

ferent size alleles were recorded and analyzed using GENOTYPER version 2.1 and GENESCAN version 3.1 software (Applied Biosystems, Foster City, CA). The presence of LOH was determined by at least two independent observers. For a given informative marker LOH was defined by a decrease of either peak of at least 50%. The results were read on computer printouts [16]. Each LOH experiment was repeated at least two times, using the same DNA preparations to evaluate the reproducibility of the results obtained.

mtDNA (D310 marker) analysis

The amplification and analysis of the mitochondrial microsatellite marker D310 was performed using a PCR-based assay as described [15]. Briefly, the DNA was amplified using a radioactively labeled forward primer (5'-ACAATTGAATGTCTGCACAG CCACTT-3') and reverse primer (5'-GGCAGAGATG TGTTTAAGTGCTG-3'). The PCR conditions are: 95°C for 2 min, 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and 72°C for 5 min. The PCR product was visualized using a 6% denaturing polyacrylamide gel.

Results

We have performed DL on a total of nine *BRCA1* carriers and five true-negatives. Of these, seven had adequate material for cytologic analysis and only one, a *BRCA1* mutation carrier, demonstrated focal minimal atypia. The remainder had benign findings. A total of 26 DL specimens were obtained (Table 1).

LOH studies at the *BRCA1* locus were possible in 19/26 DL samples, and at the *FHIT* locus in 16/26 samples. In 4/9 mutation carriers we found LOH at the *BRCA1* locus, and in two of these we also found LOH at the *FHIT* locus. Figure 1 shows an example of a DL case with LOH at the *BRCA1* and the *FHIT* genes. In one of the mutation carriers with LOH at the *BRCA1* and *FHIT* loci, minimal cytologic atypia was noted. This patient was diagnosed with invasive breast cancer in the contralateral breast, and is described in detail later in this section. In the other three carriers, either few cells were obtained, and thus the specimen was inadequate for cytologic evaluation, or the DL yielded cytologically benign cells. In one of the true negatives LOH at *BRCA1* and *FHIT* loci were also seen.

The DL specimens were also evaluated for mtDNA mutations at the microsatellite marker D310, located at

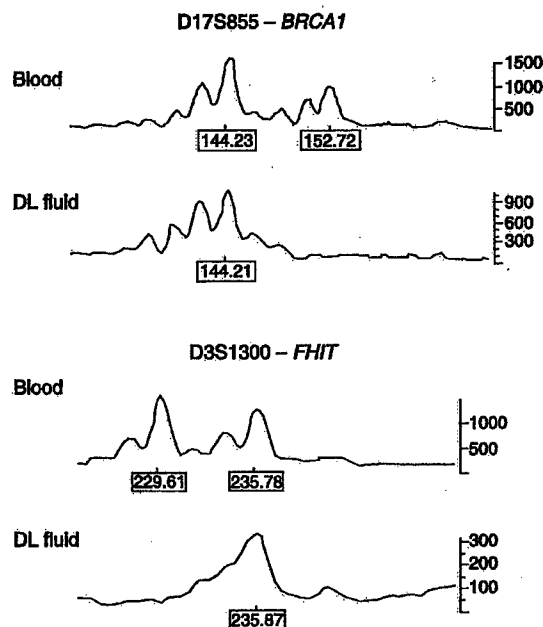


Figure 1. LOH analysis at the *BRCA1* (top) and the *FHIT* (bottom) loci, in DL fluid from a *BRCA1* carrier, showing LOH at both loci.

the D-Loop region using a PCR based assay. The mitochondrial studies were possible in all 26 DL samples. In two of the carriers in whom we detected LOH at the *BRCA1* locus, D310 length difference between the DL samples and the corresponding blood sample (somatic mutation) was found. In addition, mtDNA mutation was also detected in one additional carrier on whom we were unable to perform LOH analyses. No mtDNA mutations were detected in the true-negative subjects. Figure 2(A) shows examples of DL samples with no mtDNA mutations and Figure 2(B) shows an example of a DL sample with a mtDNA mutation.

We also evaluated six NAF samples from four patients (two carriers and two non-carriers) for mitochondrial mutations (Table 1). The evaluation was possible in 4/6 samples. A mutation was detected in one sample from a carrier whose DL from the same duct also showed the same mutation (Figure 2(B)). Three samples had no evidence of mutations, and no mutations was seen in their corresponding DL samples either. In all four NAF samples, the mtDNA findings were consistent with the corresponding DL findings.

In one *BRCA1* carrier with a recent unremarkable mammogram and physical exam, FDG-PET scan demonstrated a focal area of increased uptake in her right breast. This was subsequently biopsied and found

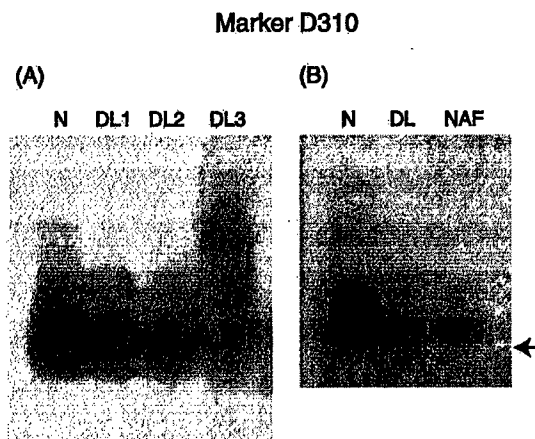


Figure 2. Analysis of the D310 marker in DL samples from two different patients. (A) shows the analysis of the D310 marker in three DL samples obtained from three different ducts from the same patient, a non-carrier of the *BRCA1* mutation (lanes DL1, DL2, DL3). No mtDNA mutation was found. Lane N shows the D310 pattern in the blood. (B) shows a mutation (arrow) at the D310 marker observed in a DL sample (lane DL) and in the NAF sample (lane NAF) from another case, a *BRCA1* carrier. Lane N shows the D310 pattern in the blood of that patient.

to be an invasive and non-invasive ductal carcinoma. In this carrier, LOH at the *BRCA1* locus was seen in DL specimens obtained from both her right (affected) and left breast. The DL specimen from her right breast had insufficient material for cellular analysis. On the left, focal minimal atypia was seen. The patient underwent bilateral mastectomy. LOH studies of the tumor tissues showed LOH at the *BRCA1* locus as was seen in the DL sample from the same breast (Table 1, Patient No. 8). Figure 3 shows LOH studies at the *BRCA1* locus performed on the DL fluid and on the breast tumor. Concordance between the two studies was noted. No evidence of mtDNA mutations was detected in the tumor or the DL samples from this patient. Careful evaluation of the non-affected breast by the pathologist showed no abnormalities. The other patients with LOH or mitochondrial abnormalities in the DL fluid had normal physical exam, a normal mammogram and normal findings on FDG-PET scan. The patients continue to be closely monitored. Of note, we were able to perform the LOH analyses and to evaluate mtDNA mutation in participants for whom DL yielded adequate cellularity, and more interestingly, in those in whom the DL was acellular. These findings indicate that the acellular specimen can still yield worthwhile and informative material for analysis.

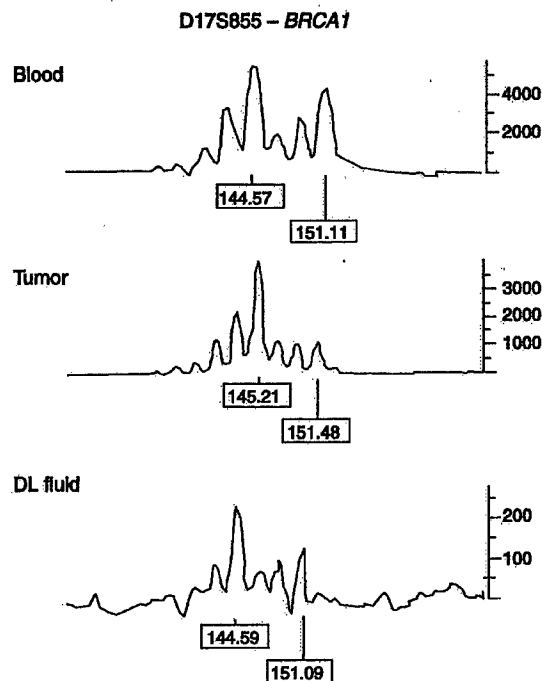


Figure 3. LOH analysis at the *BRCA1* locus in the tumor and the DL fluid from the same breast showing the same LOH in both.

Discussion

It is widely believed that the accumulation of genomic aberrations occurs very early in the process of mammary tumorigenesis and may precede morphologic changes [16–18]. Therefore, development of methods which permit the reliable identification of these early genetic changes in mammary cells obtained through non-invasive or minimally invasive approaches such as DL or nipple aspiration, will greatly benefit early detection of breast cancer.

Studies have shown that LOH at relevant polymorphic markers is present in hyperplasias (usual ductal hyperplasia and atypical ductal hyperplasia) from both cancerous and non-cancerous breasts [11, 12, 19], as well as in morphologically normal lobules, adjacent to sporadic breast cancer [18]. In a recent study, we have demonstrated the presence of LOH in morphologically normal tissues and in benign tissues from breast cancer patients that are carriers of mutations in the *BRCA1* and *BRCA2* genes. Such changes may represent the earliest detectable genomic aberrations that occur during the development and progression of breast cancer in these high-risk patients. Our conclusion from that study was that LOH at the

relevant *BRCA* loci is an early event in *BRCA* mutation carriers, and may be detected in non-malignant cells [16]. Recent studies have also shown the presence of several mtDNA mutations in a variety of tumor types [20]. Furthermore, these mitochondrial mutations were shown to be readily detectable in the bodily fluids [13]. We have found that a mtDNA mutation at the microsatellite marker D310 is altered in about 30% of breast cancers [15], and is detectable in breast fine needle aspirate fluid [14]. Taken together, a strategy allowing the detection of LOH at relevant markers or mtDNA mutations in the ductal fluid samples will provide an important early detection approach that could complement conventional screening.

Since it was first described in 2001 by Dooley et al. [5], there has been a growing interest in evaluating the potential use of the DL fluid as a tool for early diagnosis of breast cancer particularly in high-risk patients. Detection of breast cancer cells in DL fluid by methylation specific PCR has been reported [6], and evaluation of the cytologic preparations of the DL fluid cells, using fluorescence *in situ* hybridization, have also been reported [8]. In this report, we show the feasibility of using the free DNA isolated from the DL fluid and the NAF to study LOH and to detect mtDNA mutations at the microsatellite marker D310. This approach can potentially be performed on lavage samples with low cellular content or no distinguishable intact cells, and offers the advantage of preserving the total cellular content of the ductal fluid specimen for cytologic evaluation. Previous studies with long term follow up have shown that women with atypical ductal epithelial cells in the NAF and the fine needle aspirate fluid, have increased risk for developing breast cancer [4]. More recently, in a large multicenter study, cytologic atypia was detected in 24% of samples obtained by DL from high-risk women [5]. In that same study, 22% of the samples had insufficient cellular material for diagnosis. Therefore, the ability to isolate and use the free DNA present in the ductal fluid is very critical, particularly for the NAF samples where the cellular content of the specimen is very low, averaging 120 cells, and for the DL samples with insufficient cells for cytologic analysis. In summary, our data shows that free DNA can be isolated from breast ductal fluid obtained by nipple aspiration or DL and used for molecular studies. Thus this approach can potentially be considered in a strategy for risk assessment for breast cancer development, particularly in high risk-groups where such strategies are critically needed.

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